DESCRIPTION

TITLE OF THE INVENTION

Thermostable L-arabinose isomerase and process for preparing D-tagatose

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing the construction strategy of an expression vector containing arabinose isomerase gene of the invention.

FIG. 2 is a graph showing activity profile of arabinose isomerase of the invention depending on temperature.

FIG. 3 is a graph showing thermostability of arabinose isomerase of the invention.

BACKGROUND OF THE INVENTION

OBJECT OF THE INVENTION

FIELD IF THE INVENTION & PRIOR ART

The present invention relates generally to production of an enzyme for use in production of a sweetener. More particularly, the present invention relates to production of arabinose isomerase and tagatose.

In recent years, growing concerns about health have led much research effort to the development of healthful

foods. As one of the above efforts, sugar alcohols have been proposed as sweeteners which can substitute sugar, known to cause adult diseases, and are practically being used. Since the said sweeteners are known to have adverse side effects such as causing diarrhea when ingested more than certain amount, there is an urgent need to develop substitutional sweeteners without harmful effects.

Among substitutional sweeteners which have little side effect, tagatose, a keto-sugar of galactose, has similar sweetness to D-fructose, and has known not to be absorbed or metabolized in the body, making tagatose a safe low-caloric substitutional sweetener for sugar. Also, it has been reported that tagatose can be employed as an intermediate for the preparation of useful optically active isomers, detergents and cosmetics, also, as an additive or raw material for the synthesis of drugs, especially, its ability to lower blood sugar level renders tagatose a therapeutic and preventive agent for diabetes, and a low caloric diet agent.

Currently, tagatose is mostly prepared via chemical synthesis from galactose (see: U.S. Pat. No. 5,002,612), which comprises the steps of isomerization of galactose catalyzed by metal hydroxide in the presence of inorganic salts to form an intermediate of metal hydroxide-tagatose

complex, and neutralization of the complex by adding acid to yield final product, tagatose.

Alternative method for manufacturing tagatose is an enzymatic method in which galactose is converted into tagatose via conversion of aldose or aldose derivatives into ketose or ketose derivatives. Especially, it has been reported that arabinose isomerase which catalyzes the conversion reaction of L-arabinose into L-ribulose can be employed for production of tagatose in vitro using galactose as a substrate. However, the yield of tagatose produced by arabinose isomerase from galactose is as low as 20%, hindering industrial application of conversion process of galactose into tagarose. Although the method for manufacturing tagatose from milk or cheese has been developed (see: U.S. Pat. No. 6,057.135), again, low yield is the limitation for its industrial use.

Under the circumstances, there are strong reasons for exploring and developing a novel enzyme which can produce tagatose with high yield.

SUMMARY OF THE INVENTION

An aspect of the present invention provides an isolated polynucleotide coding for an arabinose isomerase from *Thermotoga neapolitana* 5068.

Another aspect of the present invention provides an arabinose isomerase from the above polynucleotide.

Another aspect of the present invention provides an expression vector, which comprises the above-described isolated polynucleotide.

Another aspect of the present invention provides a host cell transformed with the above-described expression vector. The host cell is *E. coli*.

Still another aspect of the present invention is a method for manufacturing tagatose from galactose by using the above-described arabinose isomerase.

DETAILED DESCRIPTION OF EMBODIMENTS

The present inventors have made an effort to develop an enzyme which can produce tagatose with high yield, and have found that tagatose can be produced with high yield from galactose by employing a recombinant arabinose isomerase produced from *E. coli* transformed with recombinant vector containing arabinose isomerase gene derived from *Thermotoga neapolitana* 5068.

To prepare thermophilic or thermostable arabinose isomerase for industrial use, the present inventors have cloned a gene coding for arabinose isomerase from genomic

DNA of *Thermotoga neapolitana* 5068 (DSM 5608) and analyzed nucleotide sequence. The nucleotide sequence encoding arabinose isomerase of an embodiment of the present invention has shown to have 83.2% homology to those of the putative arabinose isomerase gene of *Thermotoga maritima* of which entire nucleotide sequence has been verified via genome project.

For high level expression of the said cloned arabinose isomerase in *E. coli*, the gene coding for the enzyme was inserted into an expression vector pET22b(+) (Novagen, U.S.A.) to construct a recombinant expression vector pTNAI, which was then introduced into *E. coli* BL21. The transformed recombinant *E. coli* was named "*E. coli* BL21/DE3" and deposited with an international depository authority, the Korean Culture Center of Microorganisms (KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea) on Dec. 4, 2000 as accession no. KCCM-10231.

The said *E. coli* BL21/pTNAI was grown to obtain recombinant arabinose isomerase, which was characterized to have optimum pH of 5.0-8.0, optimum reaction temperature of 90 ℃ Furthermore, over 80% of remaining activity was measured after 2 hour heat treatment at 70 ℃, indicating that the enzyme is exceedingly heat stable.

Aqueous solution of galactose was subjected to isomerization reaction employing recombinant arabinose isomerase of the embodiment of the present invention, and it has been found that conversion rate into tagatose was from 40% to 50%.

When the said recombinant arabinose isomerase is employed for industrial production of tagatose, soluble form of the enzyme may be employed, nevertheless, it is more preferable to immobilize the enzyme on the beads used in industry.

EXAMPLES

Embodiments of the present invention are further illustrated in the following examples, which should not be taken to limit the scope of the invention.

[Example 1]

Cloning of Arabinose Isomerase Gene

Thermotoga neapolitana 5068 (DSM 5068) was grown under an anaerobic condition and cells were harvested by centrifugation at 8000×g for 10 minutes. Genomic DNA isolated from the cells harvested above was partial digested with Sau3AI (TaKaRa Biotechnology, Japan) to obtain 12 kb or shorter fragments of DNA. The DNA fragments were inserted into ZAP Expression Vector (Stratagene, U.S.A.) and packaged to prepare a genomic library of

Thermotoga neapolitana 5068. Nucleotide sequences of the genes for conventional thermophilic or thermostable arabinose isomerase were analyzed to prepare primer araAF: 5'-ATGATAGATCTCAAGCAGTAC-3' (SEQ ID NO: 1) and primer araAR: 5'-TCATCTTTTCAAAAGCCCCC-3' (SEQ ID NO: 2), which were used in PCR for the preparation of probes for DNA-DNA hybridization. The genomic library prepared above was screened for DNA fragments containing arabinose isomerase gene by DNA-DNA hybridization to obtain a recombinant vector containing a gene encoding arabinose isomerase of *Thermotoga neapolitana* 5068. The nucleotide sequence of arabinose isomerase gene (SEQ ID NO: 3) cloned above and the deduced amino acid sequence (SEQ ID No: 4) from the said gene were compared with those of known arabinose isomerase genes, respectively (see: Table 1).

TABLE 1 Comparison of homology between arabinose isomerase of one embodiment of the present invention and known arabinose isomerases

Strain	Gene Sequence (homology, %)	Amino Acid Sequence (homology, %)
Thermotoga maritima	83.2	94.8
Bacillus stearothermophilus	61.9	62.8
Bacillus halodurans	59.1	59.0
Bacillus subtilis	58.6	55.5
Salmonella typhimurium	57.8	54.5
Escherichia coli	59.0	54.3

Mycobacterium smegmatis	56.3	50.7

As shown in Table 1, it has been found that the arabinose isomerase of the embodiment of the present invention is a novel enzyme which has 83.2% homology of nucleotide sequence and 94.8% homology of amino acid sequence to the sequences of published putative arabinose isomerase of *Thermotoga maritima*, respectively.

[Example 2]

Preparation of Recombinant Expression Vector and Recombinant *E. coli*

In order to obtain high level expression of the said thermostable arabinose isomerase in *E. coli* using the arabinose isomerase gene obtained in Example 1, the said gene was inserted into an expression vector pET 22b(+) (Novagen, U.S.A.) double-digested with NdeI and EcoRI to construct a recombinant expression vector pTNAI (see: FIG. 1), which was then introduced into *E. coli* BL21. The transformed recombinant *E. coli* was named "*E. coli* BL21/DE3" and deposited with an international depository authority, the Korean Culture Center of Microorganisms (KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea) on Dec. 4, 2000 as accession no. KCCM-10231.

[Example 3]

Expression of Recombinant Arabinose Isomerase

The recombinant *E. coli* BL21/DE3 prepared in Example 2 was inoculated into LB (Luria-Bertani) medium (1% v/v) and incubated at 37 ℃ for 2 hours, to which lactose was added to a final concentration of 1 mM and expression of recombinant arabinose isomerase was induced for 12 hours. For assay of expressed arabinose isomerase, cells were collected by centrifugation at 8000.times.g for 10 minutes, resuspended in 10 ㎖ of 100 mM MOPS buffer (4-morpholinepropanesulfonic acid, pH 7.0), and then disrupted by sonication to obtain crude enzyme, with which arabinose isomerization reaction was carried out. Arabinose isomerization was performed by mixing 100 ㎕ of the said crude enzyme solution with 10 mM (final concentration) galactose as a substrate, followed by adding 1 ml of enzyme reaction buffer (100 mM MOPS buffer, pH 7.0) containing cofactors (5 mM $MgSO_4$, 5mM $MnCl_2$, 0.5mM $CoCl_2$) and incubating at 80 ℃ for 30 minutes. The product of the above reaction was detected using cysteine-carbazole-sulfuric acid method (see: Dische, Z., and E. Borenfreund., A New Spectrophotometric Method for the Detection and Determination of Keto Sugars and Trioses, *J. Biol. Chem.*,

192:583-587, 1951), and it has been found that normal arabinose isomerization has been undergone.

[Example 4]

Purification of Recombinant Arabinose Isomerase

For purification of recombinant arabinose isomerase expressed by the method described in Example 2, cells were collected by centrifugation at 8000×g for 19 minutes and cell wall of *E. coli* was disrupted by sonication, which was followed by centrifugation at 20,000×g for 20 minutes to obtain supernatant. Then, the said supernatant was heat-treated at 90 ℃ for 60 minutes, centrifuged at 20,000×g for 20 minutes to get rid of precipitate, and the supernatant was further purified by ammonium sulfate-mediated precipitation and finally ion-exchange column chromatography (Q-Sepharose Fast Flow, Pharmacia, Sweden). pH dependency of the said purified enzyme was analyzed and optimum pH was found to be 5.0 to 8.0.

[Example 5]

Optimum Temperature of Recombinant Arabinose Isomerase

Optimum temperature for purified recombinant arabinose isomerase prepared in Example 4 was determined using the same method as described in Example 3. The tested reaction temperatures for arabinose isomerase were 50, 60,

70, 80, 90 and 100 ℃ and maximum activity was obtained around 85 ℃(see: FIG. 2).

[Example 6]

Thermostability of Recombinant Arabinose Isomerase

To assess the thermostability of recombinant arabinose isomerase of the embodiment of the present invention, crude enzyme prepared in Example 3 was heat-treated at 90 ℃ for 10, 30, 60, 90 and 120 minutes respectively, and remaining activity of recombinant arabinose isomerase for isomerization was determined as described in Example 3 (see: FIG. 3). As shown in FIG. 3, it has been found that over 80% of enzyme activity was remained after 2 hour heat-treatment at 90 ℃.

[Example 7]

Production Yield for Tagatose at Various Temperature

By employing recombinant arabinose isomerase of the embodiment of the present invention, the production yield for tagatose was determined at various temperatures. 1 ㎖ of enzyme reaction mixture in Example 3 was reacted at 60, 70, 80 and 90 ℃ for 120 minutes, tagatose yield was determined employing BioLC (see: Table 2).

TABLE 2 Production Yield for tagatose at various temperatures

Enzyme Reaction Temperature	60℃	70℃	80℃	90℃
Production Yield for Tagatose	44.7	46.5	48.3	49.7

As shown in Table 2, the higher the reaction temperature was, the higher tagatose yield was obtained, and production yield for tagatose was as high as 40-50%.

As clearly illustrated and demonstrated above, the present invention provides, among other things, a gene coding for thermostable L-arabinose isomerase derived from *Thermotoga neapolitana* 5068, a recombinant vector containing the said gene, a microorganism transformed with the said vector, a thermostable arabinose isomerase from the said transformant and a process for preparing D-tagatose employing the said enzyme. Since the recombinant arabinose isomerase of the embodiment of the present invention is highly thermostable and can produce tagatose with high yield at high temperature. it can be efficiently applied in pharmaceutical and food industries.